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Phosphatidylcholine and sphingomyelin containing an *elaidoyl* fatty acid can form cholesterol-rich lateral domains in bilayer membranes

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Abstract

Elaidic acid is a *trans*-fatty acid found in many food products and implicated for having potentially health hazardous effects in humans. Elaidic acid is readily incorporated into membrane lipids *in vivo* and therefore affects processes regulating membrane physical properties. In this study the membrane properties of sphingomyelin and phosphatidylcholine containing elaidic acid (*N*-E-SM and PEPC) were determined in bilayer membranes with special emphasis on their interaction with cholesterol and participation in ordered domain formation. In agreement with previous studies the melting temperatures were found to be about 20 °C lower for the elaidoyl than for the corresponding saturated lipids. The *trans*-unsaturation increased the polarity at the membrane–water interface as reported by Laurdan fluorescence. Fluorescence quenching experiments using cholestatrienol as a probe showed that both *N*-E-SM and PEPC were incorporated in lateral membrane domains with sterol and saturated lipids. At low temperatures the elaidoyl lipids were even able to form sterol-rich domains without any saturated lipids present in the bilayer. We conclude from this study that the ability of *N*-E-SM and PEPC to form ordered domains together with cholesterol and saturated phospho- and sphingolipids in model membranes indicates that they might have an influence on raft formation in biological membranes.

Keywords: Phosphatidylcholine; Sphingomyelin; Cholestatrienol; *Trans*-parinaric acid; Fluorescence quenching; Laurdan

1. Introduction

Trans-unsaturated fatty acids are produced from their *cis*-isomers during partial hydrogenation procedures used by the food industry to harden oils. *Trans*-fatty acids are consequently found in a variety of food products. Industrially produced *trans*-fatty acids have received some attention lately due to their potentially hazardous health effects [1–4]. Like saturated fatty acids, also *trans*-fatty acids seem to cause an elevation in blood LDL, whereas *trans*-fatty acids in contrast to saturated fatty acids also lower the blood HDL [5,6]. The effect of *trans*-fatty acids on blood LDL/HDL ratio can therefore be even more severe than that of saturated fatty acids [2,5]. Epidemiological studies have also found that the consumption of *trans*-fatty acids increases the risk of coronary heart disease, sudden death and possibly also diabetes mellitus [2,4]. These reports have recently led to legislation concerning high *trans*-fatty acid food in some countries. Denmark has decided to reduce the *trans*-fatty acid content of food remarkably during the last 2 years [4]. In some countries (e.g. the US) the amount of *trans*-fatty acids in food products must be declared on nutritional information labels [7]. Although epidemiological studies have shown serious health complications associated with the intake of *trans*-fatty acids their mechanism of action is yet poorly studied at the molecular level.

It was established already in the 1970s that *trans*-fatty acids taken up from the diet are incorporated into higher lipids such as

Abbreviations: 7SLPC, 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine; CTL, cholestatrienol; β-CyD, β-cyclodextrin; DPH, 1,6-diphenyl-1,3,5-hexatriene; HDL, high-density lipoprotein; Laurdan, 6-lauroyl-2-(*N*,*N*-dimethylamino)naphthalene; LDL, low-density lipoprotein; NMR, nuclear magnetic resonance; *N*-E-SM, D-*erythro*-*N*-elaidoyl-sphingomyelin; *N*-O-SM, D-*erythro*-*N*-oleoyl-sphingomyelin; *N*-S-SM, D-*erythro*-*N*-stearoyl-sphingomyelin; PC, phosphatidylcholine; PEPC, 1-palmitoyl-2-elaidoyl-*sn*-glycero-3-phosphocholine; PGalCer, *N*-palmitoyl-galactosylceramide; PGlcCer, *N*-palmitoyl-glucosylceramide; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PSPC, 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; SM, sphingomyelin; T_m, gel–liquid crystalline transition temperature; tPA, *trans*-parinaric acid

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triglycerides, phospholipids and cholesterol esters [8–10]. The major man made *trans*-fatty acid ending up in humans is elaidic acid ($18:1^{\Delta 9t}$) and this is also the *trans*-fatty acid that has been most widely used in *trans*-fat related studies. Elaidic acid is readily incorporated into membrane lipids *in vivo* and therefore comes to function as an effector in the processes regulating membrane physical properties [11–13]. The order in the *trans*-fatty acid containing phospholipid membranes has been shown to be higher than with the *cis*-analogues [13,14]. Melting temperatures (T_m) for phospholipids are also much less affected by mono-*trans*-unsaturation compared to the corresponding *cis*-unsaturation. This can be exemplified by the phosphatidylcholines used in this study for which the T_m-values have been reported to be 49 °C for PSPC (1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine), −2.5 °C for POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) as measured by differential scanning calorimetry [11] and 26 °C for PEPC (1-palmitoyl-2-elaidoyl-*sn*-glycero-3-phosphocholine) as measured by loss of deuterium NMR signal [12]. Computer simulations have confirmed that the configuration of a *trans*-unsaturated chain in phosphatidylcholine closely resembles that of a saturated chain [15]. A *cis*-unsaturation in the middle of the acyl chain will on the other hand interfere considerably with the hydrocarbon chain packing in the core of the bilayer [16]. However, when looking at the interactions at the membrane–water interface molecular dynamics simulations show that the effect of a *trans*-unsaturated acyl chain in a PC molecule differs only slightly from that of a *cis*-unsaturated chain [17]. It seems clear from the above mentioned studies that membrane lipids containing *trans*-unsaturated acyl chains have properties that differ clearly from the corresponding saturated as well as *cis*-unsaturated lipids.

Biological membranes are heterogeneous with both transverse and lateral asymmetry of lipids. It has been shown that cholesterol and sphingomyelin co-localize in biological membranes and that they, together with glycosphingolipids and perhaps some saturated glycerophospholipids, form detergent insoluble membrane domains, also called rafts [18,19]. These rafts have been suggested to take part in cellular processes, for example signal transduction, membrane trafficking and protein sorting [18,20–23]. The domains form as a consequence of non-ideal mixing of membrane lipid components which leads to segregation of certain lipids [24]. Domains formed in bilayer membranes may then be stabilized by lipid–lipid interactions such as attractive van der Waal's forces between the saturated acyl-chains of the sphingolipids involved and possible hydrogen bonding in the head group and interfacial regions [18,20–23]. Since *trans*-unsaturated phospholipids alter bulk membrane properties, the question of how these lipids influence membrane lateral domains becomes highly relevant.

Cholesterol is localized to the plasma membranes in mammalian cells where it is the major neutral lipid component [25]. In the membrane bilayer cholesterol influences the physical state and packing density of phospholipids. The degree of intermolecular interactions between cholesterol and different phospholipids and the way the lipids are packed together in the bilayer is known to vary [26]. The interaction between cholesterol and *trans*-fatty acid containing phospholipids was recently studied by measuring the cholesterol partition coefficient between a cholesterol–cyclodextrin complex and large unilamellar vesicles containing the phospholipid [14]. The authors concluded that membranes containing *trans*-unsaturated phospholipids had a larger cholesterol affinity than corresponding *cis*-unsaturated phospholipid membranes. Studies of *trans*-fatty acid containing sphingolipids are scarce. Isolation of detergent resistant membrane domains from large unilamellar vesicles containing natural glycerophospholipids and different acyl chain defined sphingomyelin species, including *N*-elaidoyl-sphingomyelin (*N*-E-SM) together with cholesterol have been conducted. No detergent resistant domains were formed by cholesterol and *N*-oleoyl-sphingomyelin (*N*-O-SM), whereas *N*-E-SM formed detergent resistant domains but not to as large an extent as *N*-stearoyl-sphingomyelin (*N*-S-SM, [27]). In this study we wanted to explore how phosphatidylcholine and sphingomyelin containing an elaidoyl acyl chain behave in complex lipid bilayers.

2. Materials and methods

2.1. Materials

Cholesterol was purchased from Sigma Chemicals (St. Louis, MO, USA). D-*erythro*-*N*-stearoyl-sphingomyelin (*N*-S-SM) was purified from egg yolk sphingomyelin (Avanti Polar Lipids, Alabaster, AL, USA) by reverse-phase HPLC (Supelco Discovery C18-column, dimensions 250×21, 2 mm, 5 μm particle size, with 100% methanol as the mobile phase). The purity and identity of the product was verified on a Micromass Quattro II mass spectrometer (Manchester, UK). D-*erythro*-*N*-elaidoyl-sphingomyelin (*N*-E-SM) and D-*erythro*-*N*-*oleoyl*-sphingomyelin (*N*-O-SM) were synthesized from D-*erythro*-sphingosylphosphorylcholine (Avanti Polar Lipids) and *trans*-9-octadecenoic acid or *cis*-9-octadecenoic acid, respectively (Sigma Chemicals) [28]. *N*-Palmitoyl-galactosylceramide (PGalCer) and *N*-palmitoyl-glucosylceramide (PGlcCer) were synthesized from corresponding lyso derivatives (Avanti Polar Lipids) and palmitic acid anhydride (Sigma Chemicals) as described previously [29]. 1-Palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids. (7-Doxyl)-stearic acid (TCI Europe N.V., Belgium) and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids) were used for the synthesis of 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC) [30]. Octadecanoic acid (Larodan Fine Chemicals) and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids) were used for the synthesis of 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (PSPC) [30]. *Trans*-9-octadecenoic acid (Sigma Chemicals) and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids) were used for the synthesis of 1-palmitoyl-2-elaidoyl-*sn*-glycero-3-phosphocholine (PEPC) [30]. All products were purified by HPLC and characterized by mass spectrometry. Stock solutions of lipids were prepared in hexane/2-propanol (3:2 by vol), stored in the dark and warmed to ambient temperature before use. β-cyclodextrin (β-CyD) was obtained from Sigma Chemicals and stock solution prepared in H_2O to a concentration of 40 mM. The water used in all experiments was purified by reverse osmosis followed by passage through a Millipore UF Plus water purification system, to yield a product with a resistivity of 18.2 MΩcm. 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-lauroyl-2-(*N*,*N*-dimethylamino)naphthalene (Laurdan) were obtained from Molecular Probes (Leiden, The Netherlands).

Cholesta-5,7,9(11)-trien-3-beta-ol (CTL) was synthesized and purified using the method published by Fisher and coworkers [31]. CTL was purified in the dark by reverse-phase HPLC on a RP-18 column with methanol/acetonitrile (30:70, by vol) as eluent and positively identified by mass spectrometry. Cholesterol (99+% pure) was obtained from Sigma Chemicals. *Trans*-parinaric acid (tPA) was obtained from Molecular Probes (Eugene, OR, USA). CTL and tPA were stored dry under argon in the dark at −87 °C until solubilised in argon-purged ethanol (CTL) or methanol (tPA). Stock solutions of CTL and tPA were stored in the dark at

−20 °C and used within 3 days. During the experiments the fluorescent probes were always protected from light and the solvents saturated with argon to minimize the risk of oxidation.

2.2. Preparation of vesicles

Vesicles used in steady-state fluorescence measurements and in determination of steady-state fluorescence anisotropy were prepared to a lipid concentration of 50 μM. The lipid mixtures were dried under nitrogen, dissolved in benzene or hexane/2-propanol (3:2 by vol), redried under nitrogen and finally kept in vacuum at 22 °C for at least 30 min. The dry lipids were dispersed in argon-purged water and heated above the gel- to liquid-crystalline phase-transition temperature. The warm samples were briefly vortexed and sonicated for 2 min (25% duty cycle, power output 10W) with a Branson probe sonifier W-450 (Branson Ultrasonics, Danbury, CT, USA). This procedure gives liposomes with a rather large size distribution with a mean diameter of 200 nm (determined with a Malvern 4700 multiangle laser spectrometer at an angle of 90°).

In fluorescence quenching studies of sphingomyelins and glycerophospholipids, *F* samples consisted of POPC/7SLPC/variable phospholipid(s)/cholesterol (30:30:30:10, mol%) in F_0 samples POPC replaced 7SLPC. The 30 mol% phospholipid-fraction was either *N*-S-SM, *N*-E-SM, *N*-S-SM/*N*-E-SM (50:50, molar ratio), PSPC, PEPC or PSPC/PEPC (50:50, molar ratio). The samples were studied with CTL or tPA as the fluorescent probe. CTL replaced 1 mol% of cholesterol and tPA replaced 1 mol% of POPC.

In the fluorescence quenching studies examining the domain forming ability of glycosphingolipids and sphingomyelins, *F* samples consisted of POPC/7SLPC/variable sphingomyelin/variable glycosphingolipid/cholesterol, (30:30:15:15:10, mol%), in F_0 samples POPC replaced 7SLPC. The variable sphingomyelin was either *N*-S-SM or *N*-E-SM and the variable glycosphingolipid was either PGalCer or PGlcCer. The samples were studied with CTL or tPA as the fluorescent probe.

The samples prepared for steady-state fluorescence anisotropy consisted of phospholipid with 0.5 mol% of DPH as fluorescent probe. The phospholipid was either *N*-S-SM, *N*-E-SM, PSPC or PEPC.

2.3. Fluorescence measurements

The fluorescence quenching method has been described in detail in a previous study [32]. Briefly, lipid vesicles containing ordered and disordered domains were prepared, where POPC and the nitroxide labeled quencher 7SLPC made up the bulk of the disordered phase and the composition of the ordered phase varied. The fluorescence intensity in the *F* samples was compared to the fluorescence intensity in the F_0 samples giving the fraction of quenched fluorescence. The fluorescent probes residing in the ordered phase were protected from quenching by 7SLPC. The melting of ordered domains was seen as a decrease in fluorescence intensity when the probes come in closer contact with the quencher due to increased homology. Other possible explanations for increased quenching at higher temperature, such as vesicle rupture or merging, have been ruled out by cooling and re-heating the samples and thereby concluding that the process is reversible and reproducible. CTL is a fluorescent cholesterol analogue that has been shown to mimic the behavior of cholesterol quite well [31,33–36] and resides in cholesterol-rich ordered domains. It has previously been shown that tPA generally resides in ordered phases also when these are in the gel phase [29,37,38]. The quenching curves of CTL and tPA therefore indicate the extent of sterol-rich and ordered domain formation, respectively.

Steady-state fluorescence measurements were performed on a PTI QuantaMaster-1 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ, USA). The excitation slits were set to 1 nm for tPA and 5 nm for CTL, the emission slits were set to 5 nm for both fluorescent probes. The temperature was controlled by a Peltier element, with a temperature probe immersed in the sample solution. The samples were heated from 8 °C to 70 °C with a temperature gradient of 5 °C/min. The measurements were performed in quartz cuvettes and the solutions were kept at a constant stirring rate of 260 rpm during the entire experiments. Fluorescence intensity for CTL was detected with excitation and emission wavelengths of 324 nm and 374 nm, respectively. For tPA fluorescence intensity was detected with excitation and emission wavelengths of 305 nm and 410 nm, respectively. All experiments were performed in at least duplicates and curves shown are representative of reproducible experiments.

2.4. Determination of steady-state anisotropy

The vesicles were prepared as described above. The measurements were performed on a PTI QuantaMaster-1 spectrofluorimeter (Photon Technology International) operating in the T-format. The samples were heated from 8 °C to 70 °C with a temperature gradient of 5 °C/min. The DPH fluorescence was detected with excitation and emission wavelengths of 360 nm and 430 nm, respectively. The steady-state anisotropy, r, was determined as described in [39]. All experiments were performed in at least duplicates and curves shown are representatives of reproducible experiments.

2.5. Laurdan emission measurements

Sonicated vesicles containing pure phospholipids and 0.5 mol% Laurdan as a reporter molecule were prepared by probe sonication as described above. Laurdan emission spectra were recorded between 390 and 550 nm with a PTI QuantaMaster 1 spectrofluorimeter. The excitation wavelength was set at 365 nm. The generalized polarization (GP) values indicating the position of the Laurdan emission spectra were calculated as

$$GP = (I_{435} - I_{505})/(I_{435} - I_{505})$$

where I_{435} is the fluorescence intensity in the blue-shifted region (435 nm) of the emission spectrum and I_{505} is the corresponding red-shifted emission intensity (at 505 nm) [42]. The Laurdan emission measurements were recorded at indicated temperatures between 5 and 70 °C.

2.6. Cholesterol desorption

Desorption of cholesterol from mixed monolayers to β-cyclodextrin in the subphase was determined according to a previously described procedure [40]. Briefly, mixed monolayers containing 60 mol% cholesterol and either *N*-S-SM, *N*-E-SM, PSPC or PEPC were prepared at the air/water interface. The trough used was of a zero-order type with a reaction chamber (volume: 23.9 ml, area: 28.3 cm^2) separated by a glass bridge from the lipid reservoir. The monolayer was compressed to 20 mN/m at 22 °C, given time to stabilize (~5 min) and then kept at this pressure during the entire experiment. β-CyD was injected into the reaction chamber (volume not exceeding 1 ml) to a final concentration of 1.7 mM; the content of the reaction chamber was continuously stirred. The cholesterol desorption rate was calculated from the area decrease of the monolayer at constant surface pressure during the first 10 min after injection of β-CyD. All experiments were performed at least three times and the results are given as the mean± standard deviation.

3. Results

In order to define the gel–fluid phase transition temperatures of the saturated and *trans*-unsaturated phospholipids used in the study, we determined the steady-state anisotropy of DPH in multilamellar liposomes, containing 99.5 mol% of the phospholipid and 0.5 mol% of the fluorescent reporter molecule, as a function of increasing temperature (Fig. 1). The mid-point of the anisotropy transition occurred at about 23, 27, 42 and 48 °C for *N*-E-SM, PEPC, *N*-S-SM and PSPC, respectively, in fairly good agreement with previous data, where available [11,12,41]. Hence, there was about 20 °C difference in the melting of *N*-E-SM and *N*-S-SM, or PEPC and PSPC, respectively, due to the introduction of a single *trans*-unsaturation in the *N*-linked or *sn*-2 position of the respective phospholipid. The difference in melting temperature appeared not to be affected by the interfacial properties of the phospholipids. The degree of anisotropy in the bilayers entering the liquid phase (e.g. at T_m+5 °C) appeared not to be dependent on the acyl chain composition, but were on the

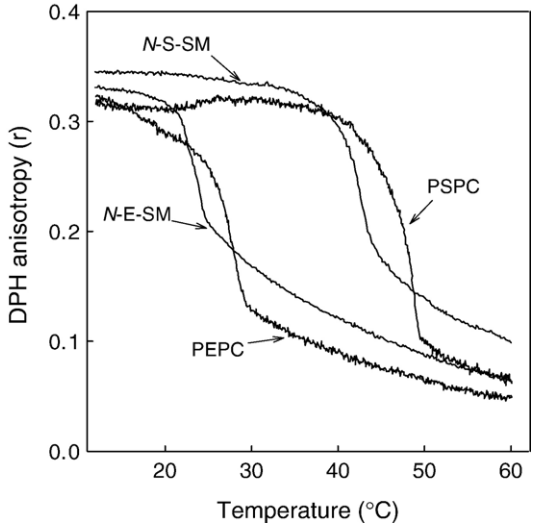

Fig. 1. Steady-state anisotropy of DPH in vesicles containing sphingomyelins or phosphatidylcholines. The vesicles were prepared from *N*-S-SM, *N*-E-SM, PSPC or PEPC, with 0.5 mol% DPH as the reporter molecule, by probe sonication at temperatures exceeding the transition temperature of the phospholipids. The lipid concentration was 50 μM and the temperature gradient was 5 °C/min.

other hand affected by the interfacial properties (higher *r* in SM and lower in PC bilayers; Fig. 1).

The polarity differences at the membrane–water interface were studied by measuring the Laurdan emission intensities in the different lipid environments and calculating the GP-values for these. The fluorescence properties of Laurdan are sensitive to the changes in polarity in the local environment of the probe at the membrane–water interface [42]. The GP values are higher in the gel than in the fluid phase bilayer due to the less polar environment of the probe in the former. Sphingomyelins have previously been shown to give more red-shifted Laurdan spectra and hence lower GP-values than corresponding phosphatidylcholines [43]. Fig. 2 shows the Laurdan GP at different temperatures in pure PEPC and *N*-E-SM vesicles comparing them to those in corresponding vesicles containing stearoyl lipids. The *trans*-unsaturation increased the polarity of the membrane–water interface. Of the four lipids studied *N*-E-SM seems to offer the most polar environment for the Laurdan probe especially at temperatures below the phase-transition. Although the order in the sphingomyelin bilayers below the melting temperature is higher in the acyl-chain region as measured by DPH anisotropy (Fig. 1) the properties at the membrane–water interface seem to resemble the fluid phase more in SM than in the corresponding PC bilayers (Fig. 2). The melting temperatures recorded as a decrease in GP-values for Laurdan are in good agreement with those reported by DPH-anisotropy.

The formation of ordered domains by the *trans*-monounsaturated phospholipids was determined in a POPC matrix using either CTL, as a marker for sterol-rich domains, or tPA, as a marker for ordered domains in general, and 7SLPC as the quencher in the sterol-poor POPC-rich domain [29,32]. Fluorescence emission intensity was measured in *F* samples consisting of POPC:7SLPC:phospholipid:cholesterol:probe (30:30:30:9:1, molar ratio) and compared to F_0 samples, in which 7SLPC was replaced by POPC. A high F/F_0 ratio thereby shows that the probe in the ordered phase is shielded from quenching and indicates the presence of ordered (as reported by tPA) and sterol-rich (as reported by CTL) domains in the bilayers. As the diffusion in the bilayer increases and the domains become smaller or more dispersed with increasing temperature the F/F_0 ratio goes down as the probes come in contact with the quencher. The latter process is what we here refer to as "domain melting". The ratio F/F_0 for different bilayer compositions is plotted as a function of temperature in Fig. 3. The top panels depict bilayers in which CTL was used, whereas tPA was used for bilayer systems shown in the bottom panels. The ordered domains formed by sterols and the saturated *N*-S-SM and PSPC were the most thermostable, with melting completed around 35–37 °C (Fig. 3A and B). The *trans*-monounsaturated phospholipids, *N*-E-SM and PEPC, also appeared to form domains that included CTL, however these domains were much less thermostable (fully melted at 18 and 14 °C, for *N*-E-SM and PEPC, respectively) as compared to the saturated counterparts. Equimolar mixtures of *N*-S-SM and *N*-E-SM, or PSPC and PEPC formed sterol-enriched domains that were intermediate in thermostability compared to the respective control situations (Fig. 3A and B).

The low melting temperature of the domains formed by the *trans*-monounsaturated phospholipids, together with the technical limitations of our instrumentation, prevented us from recording the complete melting profile of the domains. Thus the melting amplitude remains unknown for the *trans*-monounsaturated species. However, for the SM system, the mixed *N*-E-SM/*N*-S-SM domains had only half of the F/F_0 amplitude as compared to the *N*-S-SM domains (Fig. 3A and B), suggesting a lower affinity of CTL (and cholesterol) for these domains. In the

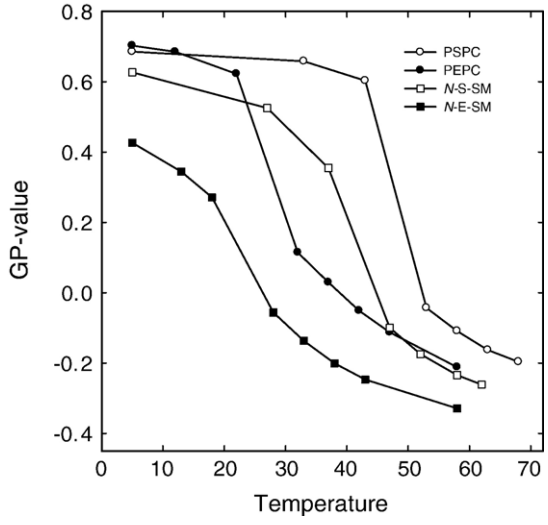

Fig. 2. Laurdan GP values measured as a function of temperature. Pure vesicles containing *N*-S-SM, *N*-E-SM, PSPC or PEPC and 0.5 mol% Laurdan as a reporter molecule were prepared by probe sonication. Laurdan emission spectra were recorded at indicated temperatures between 390 and 550 nm with a PTI QuantaMaster 1 spectrofluorimeter. The excitation wavelength was set at 365 nm and the GP values calculated from the fluorescence emission intensities in the blue-shifted region at 435 nm and in the red-shifted region at 505 nm.

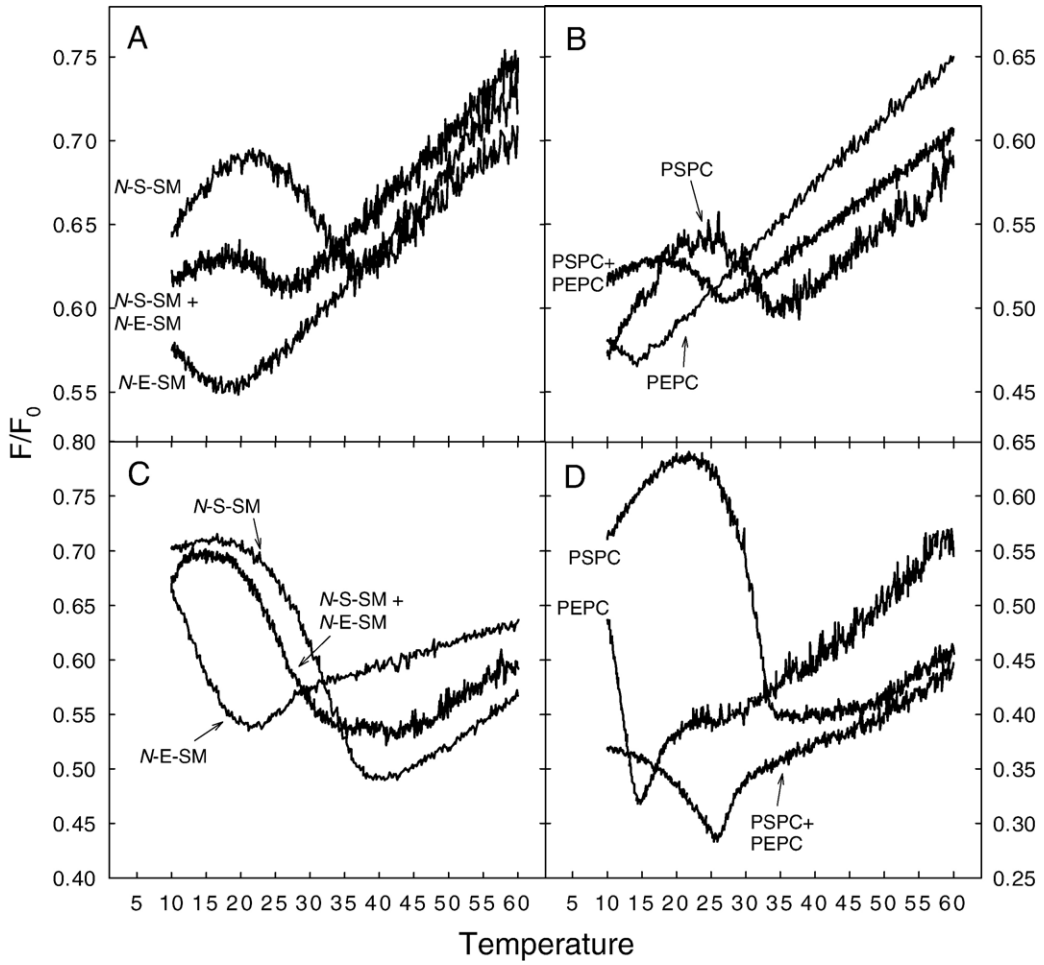

Fig. 3. Melting of phospholipid/cholesterol-rich ordered domains in a fluid bilayer as examined by quenching of CTL (A, B) and tPA (C, D). *F* samples consisted of POPC/7SLPC/variable phospholipid(s)/cholesterol (30:30:30:10, mol%) in F_0 samples POPC replaced 7SLPC. The variable phospholipid was either *N*-S-SM, *N*-E-SM, PSPC, PEPC or a combination consisting of 15 mol% of two of the phospholipids. CTL and tPA were used as reporter molecules and replaced 1 mol% of cholesterol or POPC, respectively. The total lipid concentration was 50 μM and the temperature gradient was 5 °C/min.

PC systems, the F/F_0 amplitude of the PSPC/PEPC system was also slightly diminished compared to the PSPC system.

When tPA was used to probe the thermostability of the domains formed by saturated or *trans*-monounsaturated phospholipids, very similar melting temperatures could be seen for the PC bilayers as when CTL was used (Fig. 3D and B, respectively). However, with membranes containing SMs, the tPA reported slightly higher melting temperatures as compared to CTL (Fig. 3C and A, respectively). tPA is known to be able to associate with and increase the melting temperature of ordered membrane lipids [44,45], and apparently tPA stabilized SM-domains more than PC domains.

The rate of cholesterol desorption is known to be markedly affected by the strength of cholesterol/phospholipid interaction in the donor membrane [46]. Consequently, the rate of cholesterol desorption to β-CyD can be used as a measure of how well cholesterol interacts with phospholipids in a monolayer [40]. Table 1 shows the desorption rates of cholesterol from mixed monolayers containing 60 mol% cholesterol and 40 mol % phospholipid to β-CyD in the subphase. Our results show that the cholesterol desorption from monolayers with *trans*-unsaturated species of sphingomyelin and phosphatidylcholine (*N*-E-SM and PEPC) was lower than from the *cis*-unsaturated and higher than from the saturated counterparts. Cholesterol desorption was also constantly lower from sphingomyelin

Table 1
Rate of cholesterol desorption from mixed monolayers to β-cyclodextrin in the subphase [a]

Monolayer composition (40/60 mol%)	Desorption rate (pmol cm^{-2} min^{-1})
N-S-SM/Cholesterol	2.4±0.0
N-E-SM/Cholesterol	7.3±1.1
N-O-SM/Cholesterol	10.2±0.4
PSPC/Cholesterol	8.3±1.0
PEPC/Cholesterol	13.1±1.7
POPC/Cholesterol	15.6±0.3

β-CyD was injected into the reaction chamber to a final concentration of 1.7 mM under continuous stirring. The cholesterol desorption rate was calculated from the area decrease of the monolayer during the first 10 min after injection of β-CyD. The standard deviations given were calculated from at least three experiments.

[a] Mixed monolayers were prepared at the air/water interface, compressed to 20 mN/m at 22 °C and kept at this pressure during the entire experiment.

containing monolayers compared to the corresponding phosphatidylcholines, in line with previous findings [47].

We have previously shown that while saturated cerebrosides are able to form ordered domains in complex bilayers, sterols do not appear to associate with such ordered domains to any greater extent [29]. However, when saturated sphingomyelin was included in the cerebroside domains, also sterols become associated with the ordered domains [29]. In order to study whether *N*-E-SM (or *N*-S-SM) was able to associate with cerebrosides in complex bilayers, we performed quenching experiments on the following bilayer compositions: *F* samples contained POPC/7SLPC/SM/cerebroside/sterol (30/30/15/15/10) while F_0 samples contained POPC instead of the 7SLPC. The fluorescent probe was CTL (replaced 1 mol% of cholesterol) or tPA (replaced 1 mol% of POPC). In control bilayers 30 mol% cerebroside was the sole ordered lipid.

As seen in Fig. 4A and B, CTL (and cholesterol) was not able to form sterol-enriched domains in POPC bilayers containing 30 mol% of PGalCer, in full agreement with our previous reports on similar bilayer systems [29,32]. With 30 mol% PGlcCer some sterol-enriched domains apparently formed, since there is a small discontinuity in the F/F_0 function around 30 °C (Fig. 4B—see also Fig. 1A in [29]). The presence of PGalCer or PGlcCer in the POPC matrix led to the formation of ordered domains, as reported by tPA (Fig. 4C and D), melting around 43 °C. However, inclusion of *N*-S-SM with either PGalCer or PGlcCer (equimolar mixture) led to the formation of sterol-enriched domains which readily shielded CTL from quenching by 7SLPC at low temperature (Fig. 4A and B), and which melted at 41–42 °C. Previously we reported that PSM together with either PGalCer or PGlcCer was able to form sterol-enriched domains [29]. Interestingly, *N*-E-SM was also able to loosen up the molecular packing of the cerebrosides and allow sterol to enrich in the ordered *N*-E-SM/cerebroside domains. The *N*-E-SM-containing cerebroside domains melted at a 10 °C lower temperature as compared to the *N*-S-SM/cerebroside domains (as reported by CTL—Fig. 4A and B).

Using tPA as the reporter molecule, the melting of pure PGalCer and PGlcCer domains in the POPC matrix was clearly seen (Fig. 4C and D—see also Fig. 1C in [29]). The end of

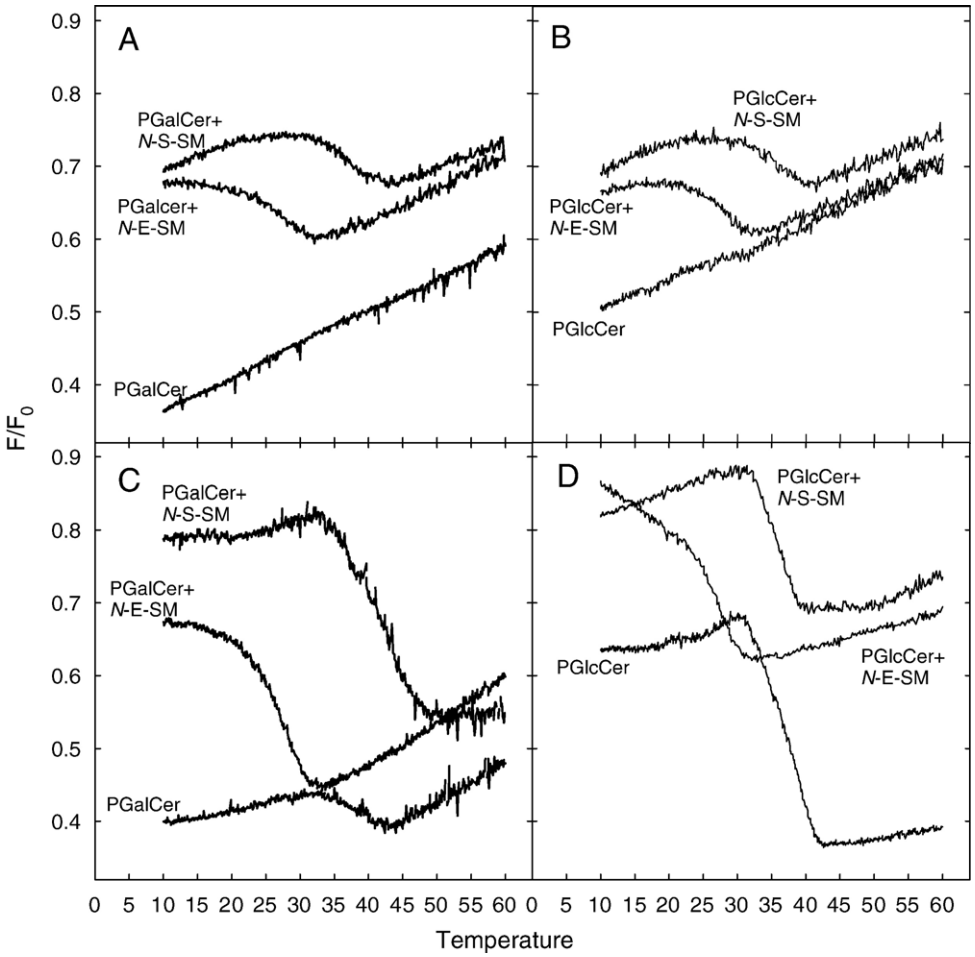

Fig. 4. Melting of ordered domains in complex glycosphingolipid containing fluid bilayers as examined by quenching of CTL (A, B) and tPA (C, D). The glycosphingolipids examined were PGalCer (A, C) and PGlcCer (B, D). The *F* samples consisted of POPC/7SLPC/ variable sphingomyelin/variable glycosphingolipid/cholesterol (30:30:15:15:10, mol%), in the F_0 samples POPC replaced 7SLPC. As reference a sample with 30 mol% glycosphingolipid (replacing sphingomyelin) was used. CTL and tPA were used as reporter molecules and replaced 1 mol% of cholesterol or POPC, respectively. The total lipid concentration was 50 μM and the temperature gradient was 5 °C/min.

melting of PGlcCer domains (with tPA) was slightly higher (a few degrees Celsius) in this study than in Maunula et al., and may relate to small differences in the concentration of the PGlcCer in these two studies. The melting temperatures for the mixed domains were rather similar as reported by CTL or tPA, except for the PGalCer/*N*-S-SM system which showed a slightly higher end of melting with tPA than with CTL as probe.

4. Discussion

This work represents a study of the properties of *trans*-unsaturated lipids in complex bilayer membranes, with special emphasis on their interaction with cholesterol and participation in ordered domain formation. Since *trans*-fatty acids have serious health implications and they are readily incorporated into phospholipids *in vivo* the properties of such lipids in membranes are of critical importance to understanding the phenomena underlying their effects. Elaidic acid, which is the *trans*-fatty acid used in this study, is produced by partial hydrogenation processes used by the food industry for cultivating oils. Elaidic acid is taken up from the diet and eventually incorporated into complex lipids in membranes.

Trans-unsaturations can be incorporated into a saturated fatty acid without affecting the tilt of the acyl chains in a bilayer structure [48]. In contrast, accommodation of *cis*-double bonds requires a change of chain tilt. Consequently *trans*-fatty acids have a more linear structural shape than the corresponding *cis*-isomers. Also when incorporated into phospholipids *trans*-fatty acids have been shown to behave like and produce membrane properties which are more similar to saturated than to *cis*-unsaturated lipids [13,14]. The melting temperatures reported here for both sphingomyelin and phosphatidylcholine were shown to be substantially lower for the elaidoyl- compared to the stearoyl-species. The *trans*-unsaturation also increased the polarity of the interface according to Laurdan GP-measurements at most temperatures studied. However, the properties of the membrane–water interface for bilayer vesicles composed of PSPC and PEPC were very similar both in liquid–crystalline and gel-phase according to Laurdan. For the sphingomyelins the environment at the interface in the *N*-E-SM vesicles seemed to always be more polar than in the *N*-S-SM bilayers. However, the properties at the interface as measured by Laurdan GP were significantly different for the *trans*-unsaturated lipids as compared to the *cis*-unsaturated (POPC or *N*-O-SM, results not shown) in contrast to what computer simulations have indicated [17].

Studies on *trans*-parinaric acid (tPA) containing four conjugated *trans*-double bonds have shown a preferential association of these fatty acids with solid phase lipids [44,45]. *Trans*-unsaturations therefore seem to allow tight enough packing of the acyl chains to favor partitioning into ordered phases. This was clearly seen for the elaidoyl-lipids in this study when domain formation was studied with tPA as the fluorescent marker. Both PEPC and *N*-E-SM formed ordered domains at low temperatures and they also participated in ordered domain formation together with the saturated counterparts. The elaidoyl chain in phosphatidylcholine has been shown by computer simulations to have a nearly identical configuration to a saturated stearic chain in the corresponding position [13,49]. In this study, the *trans*-unsaturation did not seem to affect the miscibility with the saturated counterparts to any larger extent, since these were able to form ordered domains together. *N*-E-SM was further shown to participate in ordered domain formation with the neutral glycosphingolipids (PGalCer and PGlcCer). These glycosphingolipids have high T_m values and exhibit tight lateral packing in bilayer membranes, due to their saturated hydrophobic parts and the opportunity to form hydrogen-bonding networks in the head group region [19,50]. Such neutral glycosphingolipids also have the ability to form tightly packed gel-phase microdomains in bilayer membranes [29,51]. It was therefore interesting to see that the elaidoyl-lipids were able to mix and form ordered domains together with GalCer and GlcCer.

Cis-unsaturations in the acyl chains of phosphatidylcholines and sphingomyelins have been shown to greatly interfere with their interaction with cholesterol [47,52,53]. The partitioning of cholesterol into membranes containing either a *trans*- or a *cis*-unsaturated phospholipid showed a clear preference of cholesterol for the *trans*-unsaturated membranes [14]. We show in this study that domains comprised of the elaidoyl-lipids and cholesterol can be formed in complex bilayer membranes. *N*-E-SM was even able to participate in ordered domain formation with cerebrosides in a way that promoted sterol partitioning into the domains. Our results are in good agreement with previously published results that showed detergent resistant membranes containing sterol and *N*-E-SM [27]. We also show, however, that the sterol-rich domains formed with the elaidoyl lipids will melt at substantially lower temperatures than domains formed by the saturated counterparts. Also, we see that the cholesterol desorption from monolayers to β-CyD in the subphase from monolayers containing the *trans*-unsaturated lipids will be of intermediate rate compared to that from saturated and *cis*-unsaturated monolayers, respectively. It is therefore apparent that cholesterol has an intermediate affinity for the elaidoyl lipids in the monolayers. The cholesterol interaction with sphingomyelin differs from that with phosphatidylcholines [26,47,54–56]. We can see that clearly also from the cholesterol desorption data in this study showing significantly higher desorption rates from phosphatidylcholines than from the acyl chain matched sphingomyelins.

We conclude that *N*-E-SM and PEPC participate in ordered domain formation with cholesterol and saturated phospho- and sphingolipids which suggests that they might also participate in raft-formation in biological membranes. The *trans*-unsaturation reduces the melting temperatures of both the pure lipids in bilayers and of ordered domains containing these lipids when compared to the saturated counterparts. The properties seen for these lipids in our study support the notion that *trans*-fatty acids from our diet may alter the basic properties of biological membranes substantially when incorporated into membrane lipids [5,57,58] and thereby mediate effects on membrane bound receptors [59]. The effects seen here for *N*-E-SM and PEPC on fluidity and lateral domain formation in bilayers may therefore be involved in the pathological conditions associated with *trans*-unsaturated lipids.

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